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# Expression of $\beta 1B$ Integrin Isoform in CHO Cells Results in A Dominant Negative Effect on Cell Adhesion and Motility

Fiorella Balzac, Saverio F. Retta, Adriana Albini,<sup>‡</sup> Antonella Melchiorri,<sup>‡</sup> Victor E. Koteliansky,<sup>§</sup> Massimo Geuna,<sup>\*</sup> Lorenzo Silengo, and Guido Tarone

Department of Genetics, Biology and Medical Chemistry, <sup>\*</sup>Department of Biomedical Sciences and Oncology, University of Torino; <sup>‡</sup>Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy; and <sup>§</sup>Laboratory of Physiopathology of Development, Centre National de la Recherche Scientifique 1337 and Ecole Normale Supérieure, Paris, France

**Abstract.** The integrin subunit  $\beta 1B$ , a  $\beta 1$  isoform with a unique sequence at the cytoplasmic domain, forms heterodimers with integrin  $\alpha$  chains and binds fibronectin, but it does not localize to focal adhesion sites (Balzac, F., A. Belkin, V. Koteliansky, Y. Balabanow, F. Altruda, L. Silengo, and G. Tarone. 1993. *J. Cell Biol.* 121:171–178). Here we analyze the functional properties of human  $\beta 1B$  by expressing it in hamster CHO cells. When stimulated by specific antibodies,  $\beta 1B$  does not trigger tyrosine phosphorylation of a 125-kD cytosolic protein, an intracellular signaling pathway that is activated both by the endogenous

hamster or the transfected human  $\beta 1A$ . Moreover, expression of  $\beta 1B$  results in reduced spreading on fibronectin and laminin, but not on vitronectin. Expression of  $\beta 1B$  also results in severe reduction of cell motility in the Boyden chamber assay. Reduced cell spreading and motility could not be accounted for by preferential association of  $\beta 1B$  with a given integrin  $\alpha$  subunit. These data, together with our previous results, indicate that  $\beta 1B$  interferes with  $\beta 1A$  function when expressed in CHO cells resulting in a dominant negative effect on cell adhesion and migration.

CELL adhesion and motility requires the coordinated interaction of the fibrous network of extracellular matrix proteins and the intracellular cytoskeleton (Wang et al., 1993) bridged by plasma membrane receptors of the integrin family (Hynes, 1992). Interfering with any of these three elements results in disruption of adhesive cell–matrix interactions. This can be achieved by digestion of extracellular matrix proteins with proteolytic enzymes, by disassembling the actin skeleton with specific drugs or by blocking integrins with specific antibodies. The transmembrane linkage between the extracellular matrix proteins and the cytoskeleton occurs at focal sites on the plasma membrane known as focal adhesions (Burridge et al., 1988; Geiger and Ginsberg, 1991). Immunofluorescence analysis shows that extracellular matrix proteins, integrins, and cytoskeletal proteins, specifically colocalize at focal adhesions (Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992). The molecular interactions between these components, however, is not fully understood. Integrins are heterodimers of  $\beta$  and  $\alpha$  subunits consisting of a large extracellular domain, a transmembrane domain, and a short cytoplasmic tail (Hynes, 1992). The extracellular domain of the integrin heterodimers bind to matrix proteins in

a cation-dependent manner and in many cases recognize the tripeptide sequence Arg-Gly-Asp (Ruoslahti, 1991). The cytoplasmic tails interact with cytoskeletal elements. Experimental evidence for the interaction of the cytoplasmic domain of  $\beta 1$  integrin with talin and with  $\alpha$ -actinin has been reported (Horwitz et al., 1986; Otey et al., 1990). Other molecules may be important in mediating integrin–actin interaction, these include tensin, paxillin, and zyxin (Turner and Burridge, 1991). Moreover, molecules with enzymatic or regulatory functions are localized in focal adhesions and may have a role in integrin-mediated signal transduction; these proteins include specific isoforms of protein kinase C (Jaken et al., 1989), pp60<sup>src</sup> (Rohrschneider, 1980), as well as p125FAK (Schaller et al., 1992). The latter is a cytosolic tyrosine kinase that is specifically activated when integrins are occupied by their ligands or are bound by specific antibodies (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Defilippi et al., 1994). The cytoplasmic domain of  $\beta 1$  plays a critical role in the association of integrin with the focal adhesion structure (Reszka et al., 1992) and in the activation of the p125FAK (Guan et al., 1991).

In this study we analyzed the functional properties of  $\beta 1B$ , a cytoplasmic domain variant of  $\beta 1A$ , where the last 21 COOH-terminal amino acids are replaced by a new sequence of 12 amino acids (Altruda et al., 1990). This form behaves similarly to  $\beta 1A$  in terms of fibronectin binding but has a re-

Address all correspondence to Dr. Guido Tarone, Dipartimento di Genetica Biologia e Chimica Medica, Università di Torino, Via Santena 5bis, 10126 Torino, Italy. Telephone: 39-11-670-6679; FAX: 39-11-663-4788.

stricted tissue distribution and does not localize at focal adhesion sites (Balzac et al., 1993). In this study, we show that  $\beta 1$ B does not trigger the tyrosine phosphorylation of intracellular proteins, and expression of this molecule in Chinese hamster ovary (CHO)<sup>1</sup> cells results in a dominant negative action on cell adhesion and motility.

## Materials and Methods

### Constructs and Transfections

Stable transfectants of CHO cells expressing the human integrin  $\beta 1$ A or  $\beta 1$ B were obtained as described previously (Balzac et al., 1993). Briefly, a 3.5-kb EcoRI fragment of the  $\beta 1$ B containing the entire coding sequence was inserted into the EcoRI-cloning site of the SV40-based expression vector pECE (Ellis et al., 1986). The full-length cDNA for the human  $\beta 1$ A cloned in the pECE vector was a kind gift of Filippo Giancotti (Giancotti and Ruoslahti, 1990). CHO cells were cotransfected with 20  $\mu$ g of the plasmid containing  $\beta 1$  cDNA and 2  $\mu$ g of pSV2-neo (Southern and Berg, 1982), and neomycin resistant clones were selected in HAM's F12 medium with 10% FCS and 800  $\mu$ g/ml of G418 (GIBCO BRL, Gaithersburg, MD).

### Flow Cytometry Analysis

Transfected cells were detached from culture plates by incubation in 5 mM EDTA in PBS (10 mM phosphate buffer pH 7.3, 150 mM sodium chloride) and washed twice at 4°C in PBS with 0.1 mM EDTA and 1 mg/ml of BSA. The cells were then incubated for 1 h at 4°C in the same buffer with saturating concentrations of the monoclonal antibody LM534 to the human  $\beta 1$  integrin (see below). After washing, the cells were incubated 45 min with fluorescein-labeled affinity-purified secondary antibodies (Sigma Chem. Co., St. Louis, MO), and analyzed on the flow cytometer FACS-Star Becton Dickinson (Oxford, UK), equipped with 5 W argon laser at 488 nm. Five thousand cells per sample were analyzed.

### Antibodies and Immunoprecipitation of Integrins

The following mAbs reacting with extracellular epitopes common to the two isoforms of human  $\beta 1$  were used: mAb TS2/16 (a gift from Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain); mAb G12 (a gift from Luciano Zardi, Istituto Scientifico Tumori, Genova, Italy) and mAb LM534 (a gift from Filippo Giancotti, Department of Pathology, New York University, NY). None of the above antibodies reacts with hamster  $\beta 1$ . mAb 7E2 reacting with hamster integrin  $\beta 1$ , but not with the human molecule, was a generous gift of Rudy Juliano (University of North Carolina, Chapel Hill, NC). Antibodies to cytoplasmic sequences of  $\alpha 3$ ,  $\alpha 5$ , and  $\alpha V$  integrin subunits were prepared in our laboratory and previously characterized (Defilippi et al., 1991).

Integrins were immunoprecipitated from cells labeled with <sup>125</sup>I. Labeling of membrane proteins with <sup>125</sup>I was performed as described previously (Rossino et al., 1990). Briefly, cells were released from culture dishes by 5 mM EDTA treatment in PBS and washed three times by centrifugation. Cells were suspended in PBS containing CaCl<sub>2</sub> (1 mM) and MgCl<sub>2</sub> (1 mM) and labeled with 1 mCi of <sup>125</sup>I in the presence of lactoperoxidase (200  $\mu$ g/ml) and H<sub>2</sub>O<sub>2</sub> (0.002%). For immunoprecipitation, labeled cells were extracted for 20 min at 4°C with 0.5% Triton X-100 (BDH Chemicals, Poole, Dorset, UK) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml pepstatin, and 0.1 TIU/ml aprotinin (all from Sigma Chem. Co.). After centrifugation at 12,000 g for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) and recovered by centrifugation. After washing, bound material was eluted by boiling beads in 1% SDS (Pierce, Rockford, IL) and analyzed by (6%) SDS-PAGE in the absence of reducing agents. The radioactive proteins were visualized by fluorography with sodium salicylate (Chamberlain, 1979). The amount of antibody necessary to quantitatively precipitate all labeled molecules present in the cell extract was determined in preliminary experiments. The endogenous hamster  $\beta 1$  was immunoprecipitated with mAb 7E2 while mAb LM534 was used for the transfected human  $\beta 1$ . The <sup>125</sup>I radioactivity pres-

ent in the immunoprecipitated  $\beta 1$  was determined by cutting the corresponding electrophoretic band and counting in a gamma counter.

### Western Blotting

For Western blotting integrin complexes were immunoprecipitated from unlabeled cell extracts with  $\alpha$ -specific antibodies. Immunoprecipitated material was separated by SDS-PAGE and transferred to nitrocellulose using a semi-dry apparatus (Novablot, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The blots were incubated 1 h at 42°C in 5% BSA, washed with TBS (150 mM NaCl, 20 mM Tris-Cl, pH 7.4), and incubated overnight in 10  $\mu$ g/ml  $\beta 1$  mAbs in TBS, 1% BSA. mAb G12 that specifically reacts in Western blotting with human  $\beta 1$  and mAb 7E2 reacting with hamster  $\beta 1$  were used. The blots were washed three times with TBS, incubated 2 h with peroxidase-conjugated anti-mouse Ig (Sigma Chem. Co.) and washed two times. Bound antibodies were visualized by the chemiluminescent detection method ECL (Amersham, UK). In the experiment shown in Fig. 7, the monoclonal antibodies were directly labeled with <sup>125</sup>I by the Chloramine T method. The nitrocellulose filters were first incubated with mAb G12 to visualize the human  $\beta 1$ . After stripping with 2% SDS at 42°C for 1 h to remove bound antibodies, the filter was reincubated with mAb 7E2 to visualize the hamster  $\beta 1$ . Quantitation of the band intensity was performed by densitometry using a scanner connected to a personal computer (Biomed Instrs., Fullerton, CA).

### Adhesion Experiments

Tissue culture microtiter plates were coated by overnight incubation at 4°C with the indicated concentration of purified matrix proteins and post-coated with BSA (Sigma Chem. Co.) for 1 h at 37°C. Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose according to Engvall and Ruoslahti (1977). Vitronectin was purified according to Yatohgo et al. (1988), mouse laminin from EHS tumor was obtained from GIBCO BRL (St. Louis, MO).

Cells at confluence were detached by EDTA treatment in PBS for 10 min, washed twice in serum free DMEM, and plated on the coated tissue culture plates for the indicated times. To eliminate the contribution of protein synthesis and secretion, cells were pretreated 2 h with 20  $\mu$ M cycloheximide (Sigma Chem. Co.) to prevent protein synthesis, and plated in the presence of 20  $\mu$ M cycloheximide and 1  $\mu$ M monensin (Sigma Chem. Co.) to prevent secretion (Defilippi et al., 1992). Plates were rinsed twice with PBS to remove unbound cells, and adherent cells were fixed with paraformaldehyde and stained with Coomassie blue. Cell adhesion was evaluated by reading the absorbance at 540 nm in a microtitre ELISA reader. The correspondence between the optical density and number of cells attached was confirmed in experiments showing that increasing the cell concentration in the original suspension resulted in proportional increase of the optical density.

To measure cell spreading, stained cells were photographed by choosing five random microscopic fields for each sample. The area of 150 cells per sample was determined using a computerized image analysis system (Biomed Instrs.).

### Boyden Chamber Migration Assay

Cell migration assay was carried out in Boyden chambers as previously described (Albini et al., 1987). Briefly, CHO cells and transfected clones were resuspended in serum free RPMI medium in the presence of 0.1% of BSA. Cells were then added (2  $\times$  10<sup>5</sup> cells/ml) to the upper compartment of the Boyden chamber and the lower compartment was filled with either RPMI medium containing 0.1% BSA as control, or with chemoattractants consisting of serum-free conditioned medium from 3T3 cells or of RPMI with 25  $\mu$ g/ml fibronectin. The chemoattractants were used at concentrations exerting maximal activity as evaluated in preliminary experiments. The two compartments of the Boyden chamber were separated by a polycarbonate filter (8  $\mu$ m pore size, Nucleopore, Concortezzo, Italy) coated with gelatin (5  $\mu$ g/ml, Sigma Chem. Co.). Cells were allowed to migrate 6 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells on the upper side of the filter were removed mechanically; cells on the lower surface of the filter were fixed in ethanol, stained with Toluidine blue, and 10 random fields were counted under a microscope at 160 $\times$ . Each assay was carried out in triplicate and repeated at least three times. In preliminary experiments, the ability of the cells to adhere to the filters was evaluated by fixing and staining the upper side of the filters and observation under the microscope.

1. Abbreviation used in this paper: CHO, chinese hamster ovary.

## Detection of Phosphotyrosine-containing Proteins

To analyze cell adhesion mediated by integrin monoclonal antibodies, tissue culture plates were first coated overnight at 4°C with 10 µg/ml goat anti-mouse IgG (Sigma Chem. Co.), postcoated with BSA for 1 h at 37°C and incubated with purified mAbs for 2 h at 37°C. The optimal concentration for each mAb was established by measuring cell adhesion.

Cells at confluence were pretreated 2 h with 20 µM cycloheximide (Sigma Chem. Co.) to prevent protein synthesis, detached by EDTA treatment (5 mM) in PBS for 10 min and washed twice in PBS containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>. Cells were then resuspended in prewarmed DMEM medium, in the presence of 20 µM cycloheximide and 1 µM monensin (Sigma Chem. Co.) to prevent synthesis and secretion of endogenous extracellular matrix proteins, and plated on tissue culture dishes coated with antibodies to either the endogenous (mAb 7E2) or the transfected (mAb TS2/16) β1 subunit. Cells plated on polylysine-coated tissue culture dishes were used as controls. Cells were spun at the bottom of the dishes by centrifugation at 1,000 RPM for 2 min and incubated for 5 min at 37°C. The cells were washed twice with a stop solution (5 mM EDTA, 10 mM NaF, 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.4 mM Na<sub>3</sub>VO<sub>4</sub> in PBS), and detergent extracted in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-Cl, pH 8, 5 mM EDTA, 10 mM NaF, 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.4 mM Na<sub>3</sub>VO<sub>4</sub>, 10 µg/ml leupeptin, 4 µg/ml pepstatin and 0.1 U/ml aprotinin) (all from Sigma Chem. Co.).

Protein concentration was determined in each cell extract by the Bradford protein assay method (BioRad Labs., Hercules, CA, GmbH). Samples containing equal amounts of protein were subjected to 6% polyacrylamide electrophoresis in the presence of SDS (SDS-PAGE) in reducing conditions. Proteins were transferred to nitrocellulose and processed for Western blotting as described above. The mAb PT66 to phosphotyrosine (Sigma Chem. Co.) was used followed by a peroxidase-conjugate anti-mouse IgG (Sigma Chem. Co.).

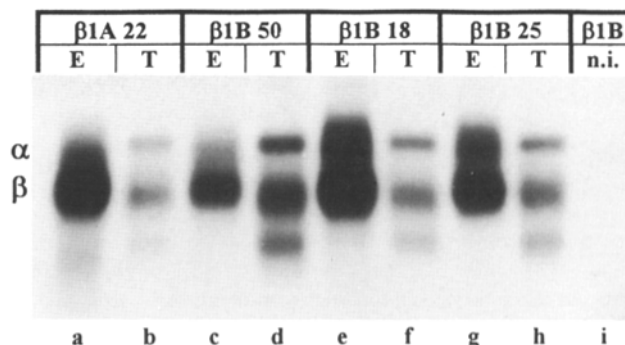
## Results

### Expression of β1A and β1B in CHO Cells Transfectants

CHO cells were transfected with the human β1B cDNA under the control of the early SV40 promoter and stable transfectants were isolated. Clones transfected with the human β1A isoform were also selected as control. CHO cells express the endogenous hamster β1A form but do not express β1B as assessed by immunoprecipitation with antibodies directed to the peptide sequence unique to β1B (Balzac et al., 1993).

To determine the expression of β1, membrane proteins were labeled with lactoperoxidase catalyzed radio iodination, and the transfected human and the endogenous hamster β1 proteins were immunoprecipitated from each clone by means of species specific monoclonal antibodies and analyzed by SDS-PAGE (Fig. 1). To quantitate the level of expression, the β1 bands were cut out of the gel and the amount of radioactivity was determined in a gamma counter. One control clone expressing human β1A (β1A-22) and three independent clones expressing β1B (β1B-18, β1B-25, and β1B-50) were selected and analyzed. As shown in Table I, clones β1B-18, β1B-25, and β1B-50 expressed increasing amounts of human β1B; the level ranges from 18 to 50% of the total β1 expressed at the cell surface (endogenous plus transfected) (Table I). The numbers in the clone name indicate the level of surface expression of the transfected protein (see below and Table I). Clone β1B-50 was previously indicated as 18.2 (Balzac et al., 1993). In the control clone β1A-22, the transfected human β1A represents 22% of the total. The ratio of transfected/endogenous β1 in β1A-22 and β1B-25 clones is comparable (Table I), moreover, the amounts of total β1 at the cell surface (endogenous plus transfected) in these clones are comparable to that present on untransfected CHO.

The relative levels of β1 expression in the different clones



**Figure 1.** Immunoprecipitation of β1A and β1B in transfected CHO cells. Clones β1A-22 transfected with human β1A and clones β1B-18, β1B-25, and β1B-50 transfected with human β1B were surface radioiodinated and extracted with detergent. The endogenous (E) and transfected (T) β1 were quantitatively immunoprecipitated, respectively, with mAb 7E2 to hamster β1 and mAb LM534 to human β1. The latter antibody recognizes an extracellular epitope common to β1A and β1B. Lane i is material immunoprecipitated with non-immune (n.i.) mouse serum. After immunoprecipitation radioactive proteins were separated by SDS-PAGE and visualized by fluorography. α and β on the left indicate the positions of the two integrin subunits. A 100-kD band of unknown identity was also immunoprecipitated from the transfected clones with antibodies to the human β1.

were confirmed by flow cytometry analysis which also showed that all clones consist of homogeneous cell population (not shown).

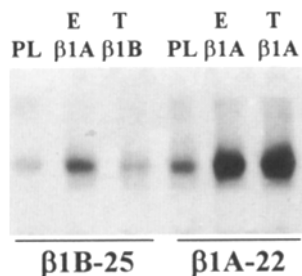
### β1B Integrin Does Not Trigger Tyrosine Phosphorylation of a 125-kD Protein

It has recently been shown that interaction of β1 integrin complexes with their ligands or with a specific antibody lead to activation of tyrosine phosphorylation of intracellular proteins (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Defilippi et al., 1994). We thus tested whether transfected β1A and β1B were equally effective in this respect. To specifically trigger the response mediated by the transfected or by the endogenous β1 subunits, cells were plated on plastic dishes coated with monoclonal antibodies specific for either human or hamster β1. Tyrosine phosphorylation of cellular proteins was determined by Western blotting with phosphotyrosine antibodies. As shown in Fig. 2, in clone β1A-22 both the endogenous and the transfected β1A integrins were equally capable to stimulate tyrosine phosphorylation of a 125-kD cytoplasmic protein. On the

**Table I.** Surface Expression of β1A and β1B in Transfected Clones

Clones	β1 Endogenous	β1 Transfected	β1 Total
β1A-22	1670*	471 (22% of total)	2141
β1B-25	1570	542 (25% of total)	2112
β1B-18	2233	498 (18% of total)	2731
β1B-50	822	825 (50% of total)	1647

\* The data are derived from an experiment shown in Fig. 1. The numbers indicate the counts per minute incorporated in the β1 bands. Values were obtained by cutting the corresponding bands on the SDS gel and counting in a gamma counter.



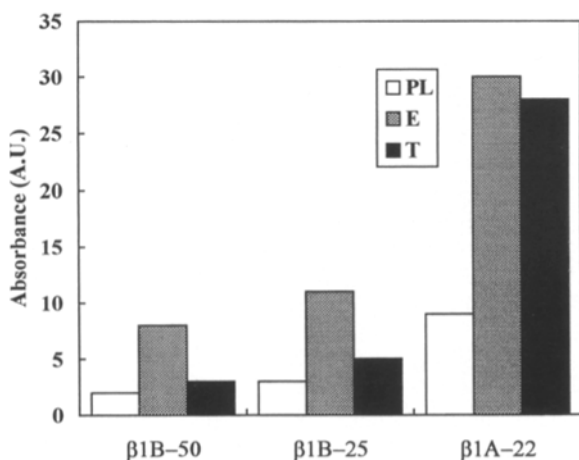
**Figure 2.** Tyrosine phosphorylation of p125 protein in  $\beta$ 1A and  $\beta$ 1B transfectants.  $\beta$ 1A-22 and  $\beta$ 1B-25 cells were plated on dishes coated with mAb 7E2 to the endogenous  $\beta$ 1 (E) or with mAb TS2/16 to the transfected human  $\beta$ 1 (T). Controls were obtained by plating cells on polylysine-coated plates (PL). After

detergent extraction equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose. Proteins containing phosphotyrosine were visualized by means of a specific monoclonal antibody (PT66) and chemiluminescence reaction. The region of the gel in the 96–160-kD range of molecular mass is shown.

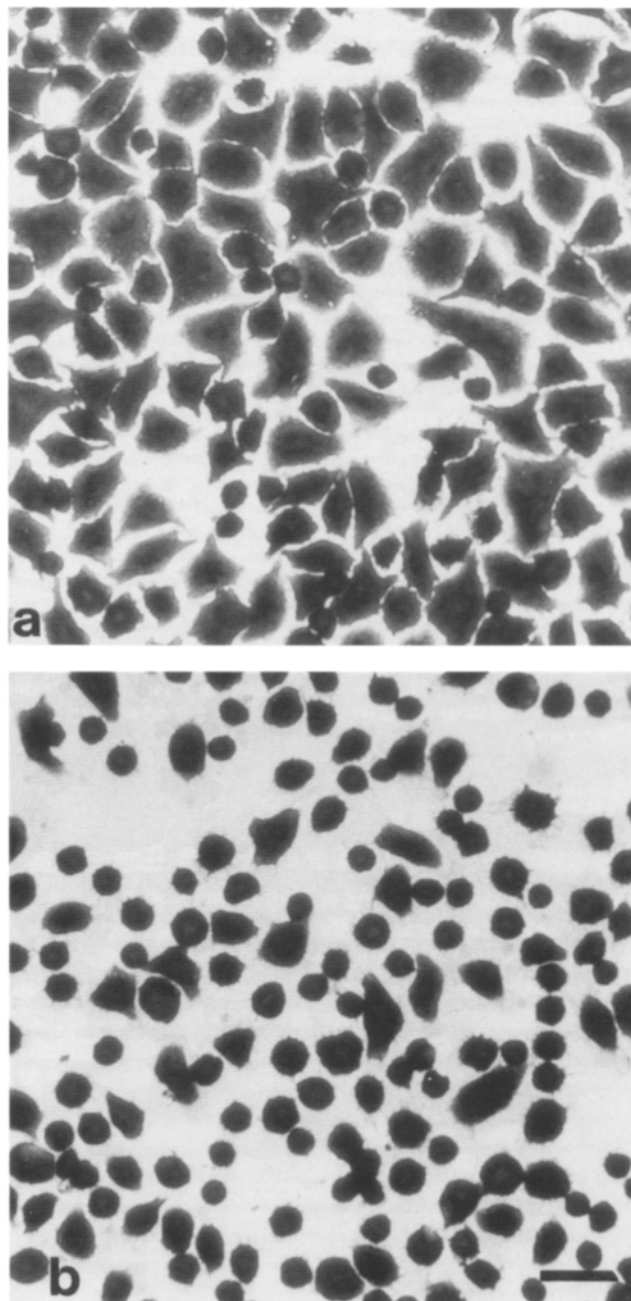
other hand, in clone  $\beta$ 1B-25, the transfected  $\beta$ 1B did not stimulate significantly the tyrosine phosphorylation of the 125-kD protein (Figs. 2 and 3). The endogenous  $\beta$ 1A, however, was active (Figs. 2 and 3), showing that the signaling pathway was not altered in this clone. The ability of the transfected human  $\beta$ 1A to stimulate tyrosine phosphorylation in clone  $\beta$ 1A-22 indicates that the human protein is active in the hamster cells and thus the lack of function of  $\beta$ 1B is related to the structural difference between the two integrin isoforms. The same phenomenon was observed in clone  $\beta$ 1B-50 (Fig. 3). Immunoprecipitation experiments showed that the 125-kD protein is specifically recognized by monoclonal antibodies to p125FAK (Schaller et al., 1992), a cytosolic tyrosine kinase localized at focal adhesions (not shown).

#### Expression of $\beta$ 1B Results in Reduced Cell Spreading

To test the adhesive response of the transfectants, cells were plated on dishes coated with antibodies specific for the transfected human protein. This allows it to directly compare



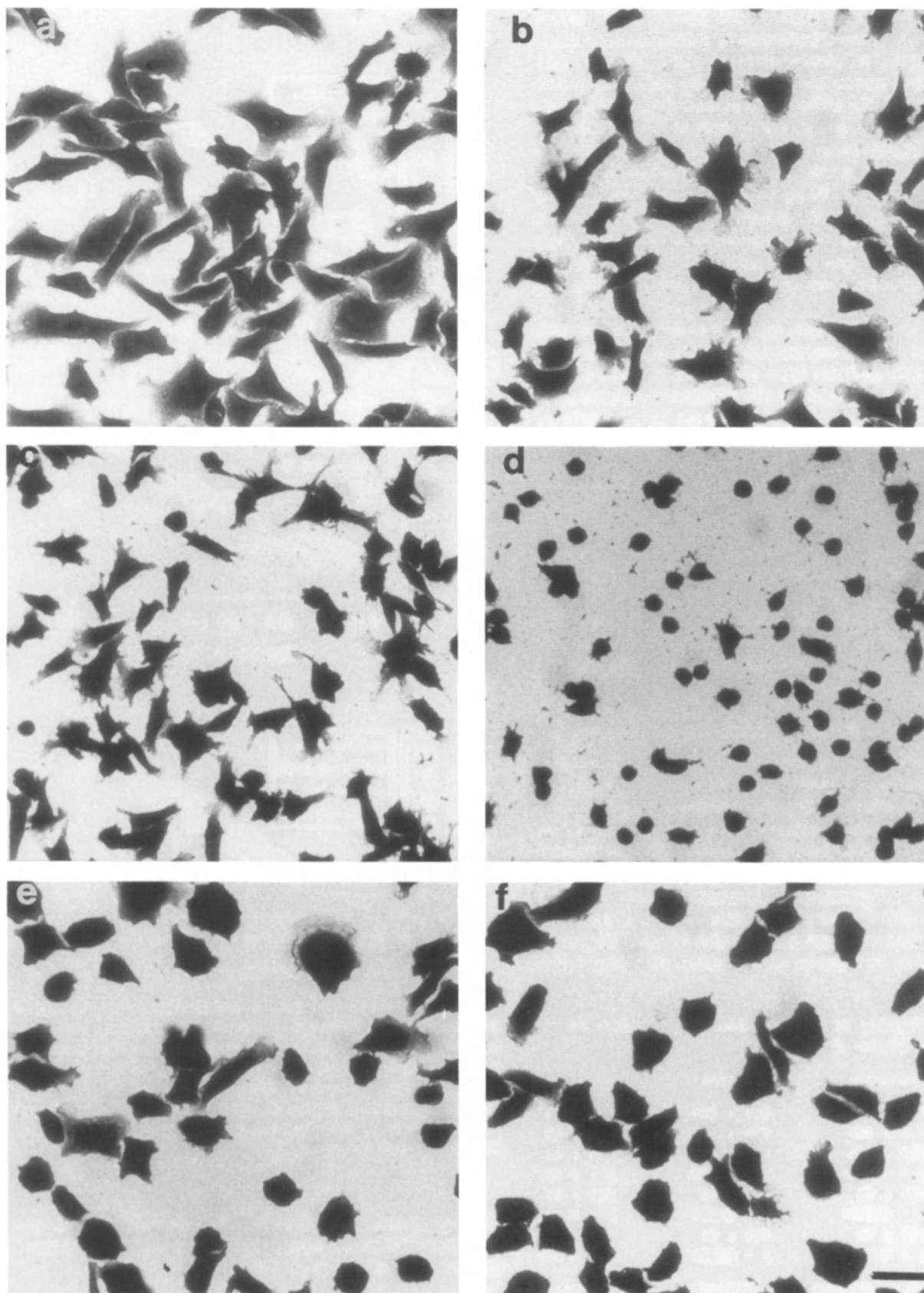
**Figure 3.** Densitometric analysis of the 125-kD tyrosine-phosphorylated proteins in  $\beta$ 1B and  $\beta$ 1A transfectants. Cells were allowed to adhere to dishes coated with polylysine (PL) or with mAb 7E2 to endogenous  $\beta$ 1 (E) or mAb TS2/16 to transfected  $\beta$ 1 (T). The proteins containing phosphotyrosine of the clones  $\beta$ 1B-50,  $\beta$ 1B-25, and  $\beta$ 1A-22 were visualized by Western blotting (see Materials and Methods). Quantitation of the intensity of the band corresponding to the 125-kD phosphoprotein was assessed by densitometry.



**Figure 4.** Adhesion of  $\beta$ 1A and  $\beta$ 1B transfectants to antibody-coated dishes.  $\beta$ 1A-22 (a) and  $\beta$ 1B-50 (b) cells were plated on dishes coated with mAb TS2/16 to the transfected human  $\beta$ 1 and allowed to adhere for 1 h at 37°C. Cells were photographed after fixation and staining. In a, cells adhere to the substratum by means of the transfected  $\beta$ 1A while in b cells use the transfected  $\beta$ 1B. Bar, 100  $\mu$ m.

adhesion mediated by either the  $\beta$ 1B or by the  $\beta$ 1A isoform. As shown in Fig. 4,  $\beta$ 1A-22 cells adhered and spread normally, while  $\beta$ 1B-50 cells failed to spread. Thus, when the  $\beta$ 1B isoform is involved in cell-substratum adhesion, cells do not spread normally.

We then tested the adhesive properties of transfected clones on dishes coated with purified matrix proteins. Under these conditions both the endogenous and transfected  $\beta$ 1 par-



**Figure 5.** Spreading of  $\beta 1A$  and  $\beta 1B$  transfectants on purified matrix proteins.  $\beta 1A$ -22 (*a*, *c*, and *e*) and  $\beta 1B$ -50 (*b*, *d*, and *f*) cells were plated on dishes coated with 2  $\mu\text{g/ml}$  of purified human plasma fibronectin (*a* and *b*); 10  $\mu\text{g/ml}$  of purified mouse laminin (*c* and *d*); and 10  $\mu\text{g/ml}$  of purified human plasma vitronectin. After adhesion for 1 h at 37°C, cells were fixed, stained, and photographed. Bar, 100  $\mu\text{m}$ .

ticipate to adhesion. To quantify cell spreading, the area of the adherent cells was determined by a computerized image analysis system. As shown in Fig. 5 and Table II,  $\beta$ 1B-expressing cells spread less on fibronectin compared to cells expressing  $\beta$ 1A. The difference was even more pronounced on laminin (Fig. 5, *c* and *d*) and in both cases it was statistically highly significant as determined with the Student's *t* test analysis. Increasing the fibronectin concentration in the coating solution from 2 to 20  $\mu$ g/ml allowed more spreading of cells but did not abolish the difference between  $\beta$ 1B and  $\beta$ 1A clones. Cell spreading on vitronectin, on the other hand, was comparable in  $\beta$ 1A and  $\beta$ 1B transfectants showing that expression of  $\beta$ 1B does not lead to a generalized spreading defect.

When the number of cells adherent to dishes coated with fibronectin or laminin was measured,  $\beta$ 1B transfectants were not significantly different from untransfected CHO or  $\beta$ 1A-22 cells (Fig. 6 *A*). Only clone  $\beta$ 1B-50 showed reduced adhesion on both substrates (Fig. 6, *A* and *B*).  $\beta$ 1B-50 cells express high levels of  $\beta$ 1B compared to the other two clones, suggesting that reduction in adhesion occurs only when  $\beta$ 1B expression exceeds a threshold level. The reduced adhesive properties of the  $\beta$ 1B-50 clone were also observed by measuring the kinetics of adhesion to a fixed amount of matrix proteins (not shown). On the other hand, all clones including  $\beta$ 1B-50 cells adhered normally when plated on vitronectin-coated dishes (Fig. 6 *C*).

In conclusion, expression of  $\beta$ 1B leads to a specific reduction in spreading of CHO cells to fibronectin and laminin, but not to vitronectin. In cells expressing the higher level of  $\beta$ 1B, cell attachment was also affected.

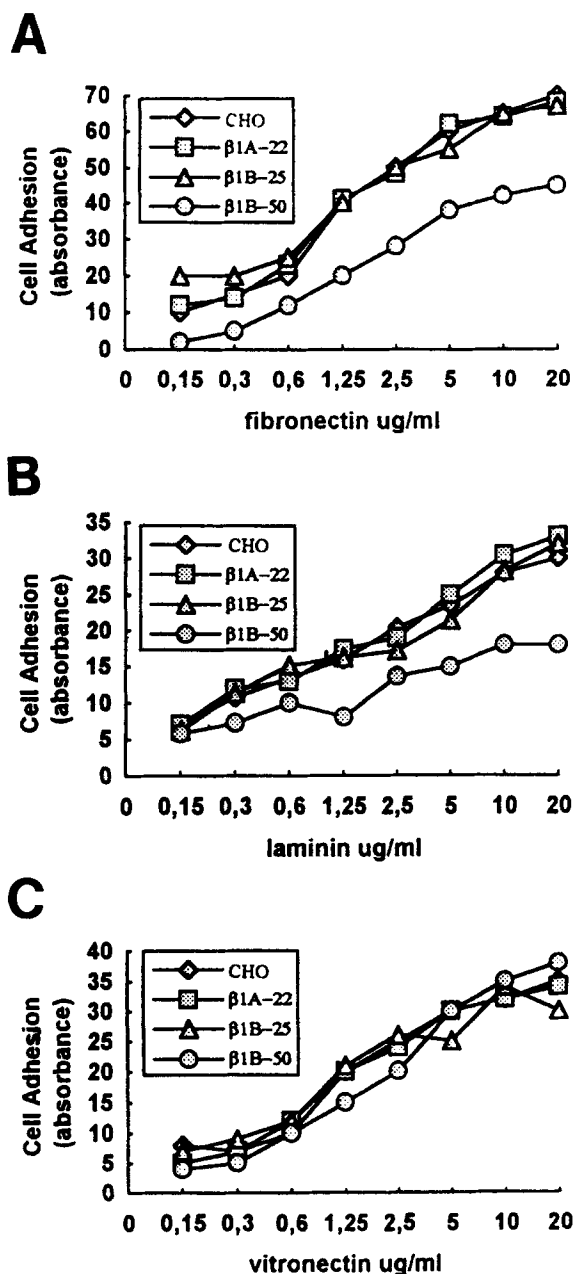
#### Expression of $\beta$ 1B Results in Reduced Cell Motility

To evaluate whether the expression of  $\beta$ 1B in CHO cells had a consequence on cell motility, we evaluated cell migration through porous filter in a Boyden chamber assay. Cells were plated on top of gelatin-coated porous polycarbonate filters and allowed to migrate toward the bottom surface. Soluble fibronectin or medium conditioned by 3T3 cells were added in the bottom compartment as chemoattractants. As shown in Table III,  $\beta$ 1B-25 and  $\beta$ 1B-50 cells showed a significant reduction of migration compared to  $\beta$ 1A-22 cells. Migration was inhibited by 75% and by 60% in clones  $\beta$ 1B-50 and  $\beta$ 1B-

**Table II. Spreading of  $\beta$ 1A and  $\beta$ 1B Transfectants on Extracellular Matrix Proteins**

Clones	Fibronectin	Laminin	Vitronectin
CHO	565 $\pm$ 130*	285 $\pm$ 105	570 $\pm$ 75
$\beta$ 1A-22	555 $\pm$ 128	276 $\pm$ 112	580 $\pm$ 90
$\beta$ 1B-25	480 $\pm$ 106	148 $\pm$ 29	574 $\pm$ 62
	<i>p</i> = 0.0084	<i>p</i> = 0.0001	<i>p</i> = 0.158
$\beta$ 1B-18	484 $\pm$ 96	156 $\pm$ 38	568 $\pm$ 52
	<i>p</i> = 0.0081	<i>p</i> = 0.0001	<i>p</i> = 0.140
$\beta$ 1B-50	477 $\pm$ 85	150 $\pm$ 54	555 $\pm$ 70
	<i>p</i> = 0.0088	<i>p</i> = 0.0001	<i>p</i> = 0.125

\* Cells were allowed to adhere to dishes coated with 2  $\mu$ g/ml of fibronectin; 10  $\mu$ g/ml of mouse laminin; 10  $\mu$ g/ml of vitronectin for 1 h. To measure cell spreading, stained cells were photographed by choosing five random microscopic fields for each sample. The area of 150 cells per sample was determined using a computerized image analysis system. Values represent the mean cell area in arbitrary units  $\pm$  SD. The *p* value was calculated with a Student's *t* test.



**Figure 6.** Adhesion of  $\beta$ 1A and  $\beta$ 1B transfectants on purified matrix proteins. Microtitre plates were coated with increasing concentrations of (A) human plasma fibronectin; (B) mouse laminin; and (C) human plasma vitronectin. Cells were suspended by EDTA treatment and plated in serum free medium for 1 h at 37°C. Cell adhesion was measured by reading the optical density after staining with Coomassie blue.  $\diamond$ , untransfected CHO;  $\square$ ,  $\beta$ 1A-22 cells;  $\triangle$ ,  $\beta$ 1B-25 cells;  $\circ$ ,  $\beta$ 1B-50 cells.

25, respectively, compared to clone  $\beta$ 1A-22. Also in this case, no differences in migration between  $\beta$ 1A and  $\beta$ 1B transfectants were observed on vitronectin.

#### Association of $\beta$ 1A and $\beta$ 1B with Hamster Integrin $\alpha$ Subunits

A possible explanation of the adhesive behavior of  $\beta$ 1B-transfected cells is that  $\beta$ 1B associates with specific integrin

**Table III. Migration of  $\beta 1A$  and  $\beta 1B$  Transfectants in Boyden Chambers**

Chemoattractant (lower chamber)	Clones		
	$\beta 1A-22$	$\beta 1B-50$	$\beta 1B-25$
Conditioned medium 3T3 <sup>†</sup>	122 $\pm$ 3*	27 $\pm$ 1	36 $\pm$ 1
FN 25 $\mu$ g/ml	92 $\pm$ 9	17 $\pm$ 4	22 $\pm$ 3
VN 50 $\mu$ g/ml	50 $\pm$ 5	50 $\pm$ 3	ND
SFM <sup>‡</sup>	8 $\pm$ 1	10 $\pm$ 2	12 $\pm$ 1

\* Mean values  $\pm$  SD of number of cells (per microscopic field) migrated on the lower surface of the porous filter.

<sup>†</sup> Conditioned medium by 3T3 cells.

<sup>‡</sup> SFM, serum free medium.

$\alpha$  subunits generating a different ratio between integrin complexes normally present at the cell surface of CHO cells. By screening with a panel of  $\alpha$  subunit specific antibodies, we determined that CHO cells express  $\alpha 3/\beta 1$ ,  $\alpha 5/\beta 1$ , and  $\alpha V$ ; the latter one forms heterodimers with both  $\beta 1$ ,  $\beta 3$ , and  $\beta 5$ .

To determine the relative amount of  $\beta 1A$  and  $\beta 1B$  associated with each  $\alpha$  subunit, we performed immunoprecipitation experiments from transfected cells using  $\alpha 3$ ,  $\alpha 5$ , and  $\alpha V$  antibodies; the immunoprecipitated material was run on SDS-PAGE and probed in Western blotting with antibodies to human  $\beta 1$  (mAb G12). The relative amount of the two  $\beta 1$  associated with each  $\alpha$  was then determined by densitometry. As shown in Table IV, the relative proportion of the human  $\beta 1A$  and  $\beta 1B$  present in the three heterodimers was comparable, confirming that the two  $\beta 1$  isoforms did not differ in their association with integrin  $\alpha$  chains. We also analyzed whether the human and the hamster  $\beta 1$  molecules showed the same pattern of association with hamster  $\alpha$  subunits. The integrin complexes were immunoprecipitated with  $\alpha$ -specific antibodies and the associated  $\beta 1$  subunits were detected by Western blotting with the antibodies specific for human (mAb G12) and for hamster  $\beta 1$  molecule (mAb 7E2) as described in the Materials and Methods section. As shown in Fig. 7, the human and hamster  $\beta 1$  subunits displayed similar association with  $\alpha 3$  and  $\alpha 5$ , while a slight preference of the human  $\beta 1$  subunits for  $\alpha V$  was observed.

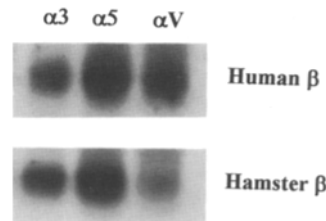
## Discussion

The cytoplasmic domains of the integrin  $\beta 1$  and  $\alpha$  subunits are highly conserved during evolution (for review see Sastry and Horwitz, 1993), while the primary sequence of the extracellular domains is much less conserved. This indicates a strong evolutive pressure to maintain a given sequence in the cytoplasmic region for the various integrin subunits and

**Table IV. Association of Transfected Human  $\beta 1$  Integrins with CHO Cell  $\alpha$  Subunits**

Clones	Association of transfected $\beta 1$ with:		
	$\alpha 3$	$\alpha 5$	$\alpha V$
$\beta 1A-22$	32*	57	11
$\beta 1B-50$	30	60	10

\* The amount of transfected  $\beta 1$  associated with the endogenous  $\alpha$  subunits was determined by densitometry after immunoprecipitation and SDS-PAGE and it is expressed as % of the total transfected protein.



**Figure 7. Association of human and hamster  $\beta 1$  with hamster  $\alpha$  subunits.**  $\beta 1B-50$  cells were immunoprecipitated with  $\alpha 3$ ,  $\alpha 5$ , or  $\alpha V$  antibodies, and the heterodimers were separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. The

coprecipitated  $\beta 1$  was detected by incubating the filter with  $^{125}I$ -labeled antibodies specific for the human  $\beta 1$  (mAb G12). After stripping with 1% SDS, the filter was reincubated with  $^{125}I$ -labeled antibodies specific for the hamster  $\beta 1$  (mAb 7E2). Similar results were obtained with cells transfected with human  $\beta 1A$ .

suggests that the molecular interactions with cytoskeletal proteins and with proteins of the intracellular signaling machinery pose rigorous structural constraints. Splice variants characterized by new cytoplasmic domain sequences have been identified for several integrin subunits including:  $\beta 1$  (Altruda et al., 1990; Languino and Ruoslahti, 1992),  $\beta 3$  (van Kuppevelt et al., 1989),  $\beta 4$  (Tamura et al., 1990),  $\alpha 3$  (Tamura et al., 1991),  $\alpha 6$  (Hogervorst et al., 1991; Tamura et al., 1991), and  $\alpha 7$  (Collo et al., 1993). On the basis of the considerations discussed above, these variant cytoplasmic sequences are expected to generate specific intracellular signaling and interact with unique cytoskeletal components.

We have identified a  $\beta 1$  variant,  $\beta 1B$ , characterized by a distinct cytoplasmic sequence (Altruda et al., 1990). Previous data have shown that  $\beta 1B$  form heterodimers with integrin  $\alpha$  chains and binds fibronectin, but it does not localize to focal adhesion sites (Balzac et al., 1993). Here we further analyze the functional properties of this variant showing that  $\beta 1B$  does not trigger the tyrosine phosphorylation of intracellular proteins and has a dominant negative effect on cell spreading and motility.

$\beta 1B$  is expressed in a tissue specific pattern and is always coexpressed with  $\beta 1A$  (Balzac et al., 1993). The coexpression of the two isoforms hampers the functional analysis of  $\beta 1B$  in cells that normally express it. To circumvent this problem we transfected human  $\beta 1B$  in hamster cells that express only the  $\beta 1A$  isoform. In this system it is possible to probe the function of the two isoforms by using species specific antibodies directed to extracellular epitopes and it is, thus, relatively simple to assign functional properties to the transfected molecule.

We have used this approach to show that  $\beta 1B$  cytoplasmic variant does not trigger increased tyrosine phosphorylation of the cytoplasmic p125-kD protein. This molecule is a major component undergoing tyrosine phosphorylation in response to integrin-ligand interaction and it corresponds to the focal adhesion kinase p125FAK (Burrage et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992). The lack of signaling through this pathway was not due to a gross defect of  $\beta 1B$  since this variant is exported at the cell surface and forms heterodimers capable of binding extracellular ligands (Balzac et al., 1993). Moreover, the endogenous  $\beta 1A$  in the same transfected cells effectively triggers tyrosine phosphorylation, ruling out the possibility that the signaling pathway is nonfunctional in these transfectants. In addition, independent transfectants showed that human  $\beta 1A$  isoform is fully functional in signal transduction in the CHO back-

ground. The lack of tyrosine phosphorylation of the p125 protein by  $\beta 1B$  is, thus, due to the properties of its cytoplasmic sequence. These results are consistent with data reported by Guan et al. (1991) showing that specific deletion of the COOH-terminal region of the chick  $\beta 1A$ , corresponding to the region spliced out in  $\beta 1B$ , abrogated the ability to stimulate tyrosine phosphorylation of the 125-kD protein.

The  $\beta 1B$  variant was also ineffective in promoting cell spreading when cell adhered to surfaces coated with antibodies specific for this molecule. This is consistent with our previous finding that  $\beta 1B$  does not localize at focal adhesions (Balzac et al., 1993) and shows that cell spreading and signaling through tyrosine kinase require an intact  $\beta 1$  cytoplasmic domain.

Our data also show that expression of  $\beta 1B$  in CHO cells has a dominant negative effect on cell spreading and motility. The inhibition of these adhesive functions is not due to undesired mutation of the selected clones, since: (a) it is common to independent clones; and (b) it is restricted to fibronectin and laminin and does not occur on vitronectin. While fibronectin and laminin are recognized by integrins of the  $\beta 1$  class, vitronectin is preferentially recognized by  $\beta 3$  and  $\beta 5$  integrins (for review see Hynes, 1992). Thus, the effect of  $\beta 1B$  is restricted to those substrata recognized by  $\beta 1$  class integrins. The  $\beta 1B$  is functionally normal as far as the association with  $\alpha$  subunits and binding to fibronectin are concerned (Balzac et al., 1993). We also show that  $\beta 1A$  and  $\beta 1B$  are indistinguishable for the association with  $\alpha 3$ ,  $\alpha 5$ , and  $\alpha V$  subunits expressed in CHO (see Table IV). Preferential association of  $\beta 1B$  with a given  $\alpha$  subunit would change the ligand specificity of the cells and lead to reduced adhesion to specific ligands. Moreover, comparative analysis of the human and hamster  $\beta 1$  proteins showed that the specie difference did not affect the association with hamster  $\alpha 3$  and  $\alpha 5$ , although a slight preference of the human  $\beta 1$  for  $\alpha V$  was detected. This, however, can not explain the distinct reduction of adhesion and migration on fibronectin and laminin. Thus, the dominant negative effect on cell adhesion is most likely explained by the fact that  $\beta 1B$  competes with  $\beta 1A$  for association with  $\alpha$  chains and binding to the extracellular matrix, but it fails to transduce mechanical or biochemical signals inside the cell.

It is interesting to note that significant reduction of cell spreading and migration is observed in cells where  $\beta 1B$  represents less than 50% of the total  $\beta 1$  at the cell surface. Whether these effects can be explained solely by the competition of  $\beta 1B$  and  $\beta 1A$  for heterodimer formation and binding to extracellular ligands is unclear. Other phenomena may contribute to the observed phenotype.  $\beta 1B$  may perturb the complex network of low affinity cooperative interactions involved in the integrin-cytoskeleton association leading to an amplified defect in the transduction of mechanical forces across the plasma membrane. Alternatively,  $\beta 1B$  may generate specific intracellular signals leading to reduced cell adhesion. These aspects deserve further investigation.

Dominant negative effects have been reported for artificially mutated forms of adhesion receptors. A  $\beta 1$  integrin with a mutation in the extracellular domain that abolished ligand binding was shown to have a dominant negative effect on cell adhesion and spreading (Takada et al., 1992). More recently, a dominant negative effect has been described for a chimeric molecule containing the cytoplasmic domain of

the  $\beta 1$  integrin (Lukashev and Pytela, 1993). Expression of a truncated form of N-cadherin, a cell-cell adhesion receptor, consisting of the sole transmembrane and cytoplasmic regions, but lacking the extracellular domain resulted in reduced cell-cell adhesion (Kinter, 1992). The truncated form presumably competes with the native molecule by binding to cytoskeletal proteins necessary to transduce the mechanical force of adhesion. A second example comes from a chimeric membrane protein containing the cytoplasmic domain of desmoglein, a desmosomal cadherin (Trojanovsky et al., 1993). This chimera causes the disruption of desmosomes and detachment of intermediate filaments from the plasma membrane.

All examples discussed above consist of proteins with a functional intracellular segment and an altered or deleted extracellular domain. The data presented here show that an adhesion receptor containing an intact extracellular domain and an altered cytoplasmic region can also cause a dominant negative effect. In the case of  $\beta 1B$ , in fact, correct extracellular interactions elicit a peculiar intracellular response that leads to anomalous adhesion. The fact that  $\beta 1B$  represents a splice variant raises the interesting question that cells may regulate their adhesive behavior by a splicing mechanism.

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